Investigation of antibiotic resistant vibrios associated with shrimp (Litopenaeus vannamei) farms

Prabina Das
Department of Marine Biosciences, Faculty of Ocean Science and Technology, Kerala University of Fisheries and Ocean Studies, Panangad, Kochi- 682506, India

T. Raja Swaminathan
Peninsular and Marine Fish Genetic Resources Centre of ICAR-NBFGR, CMFRI Campus, P.O. Number 1603, Kochi– 682018, India

Sowmya P Mohandas
Peninsular and Marine Fish Genetic Resources Centre of ICAR-NBFGR, CMFRI Campus, P.O. Number 1603, Kochi– 682018, India

J. C Anjana
Department of Marine Biosciences, Faculty of Ocean Science and Technology, Kerala University of Fisheries and Ocean Studies, Panangad, Kochi- 682506, India

K. Manjusha
Department of Marine Biosciences, Faculty of Ocean Science and Technology, Kerala University of Fisheries and Ocean Studies, Panangad, Kochi- 682506, India

P. G. Preena (preenabimal@gmail.com)
Department of Marine Biosciences, Faculty of Ocean Science and Technology, Kerala University of Fisheries and Ocean Studies, Panangad, Kochi- 682506, India

Research Article

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Abstract

For the sustainable farming of disease-free and healthy shrimps, antimicrobial usage is frequent nowadays in shrimp-cultured system. This could lead to the emergence of severe antimicrobial resistance (AMR) in the whole ecosystem. Considering the serious impact of global AMR, the present study was focused to investigate the prevalence of antimicrobial resistant vibrios among infected shrimps (*Litopenaeus vannamei*) from two brackish-water cultured farms. Diverse species of vibrios *viz*. *V. alginolyticus*, *V. parahaemolyticus, V. cholerae, V. mimicus* and *V. fluvialis* and other isolates such as *Aeromonas hydrophila*, *A. salmonicida* and *Shewanella algae* were also recovered from the shrimps on TCBS medium.Shannon wiener diversity index, H' (loge) was found to be 1.506 for the isolates from farm 1 and 1.69 for those from farm 2. *V. alginolyticus* was found to be the most resistant isolate by showing MAR index of 0.60 followed by *V. mimicus* (0.54) and *V. parahaemolyticus*(0.42). Among the 35 antibiotics of 15 different classes tested, tetracyclines, beta lactams and cephalosporins were found as the most resistant antibiotic classes. An increased altered resistance phenotype and a drastic change in MAR index were noticed after plasmid curing. Since the studied shrimp samples are of significance in food sector and plasmid-borne AMR is evident among the isolates, public health is also alarming. This baseline information will help the authorities to curb the antimicrobial use and pave the way for establishing new alternative strategies by undertaking multidimensional "One-Health" approach.

Introduction

Shrimp aquaculture is one of the fast growing food sectors and accounts for major percent of the shrimp production globally. For the past three decades, shrimp demand has been rapidly increasing and the production is expanding at a pace of about 10% per year, which is one of the fastest rates in the aquaculture industry. Shrimp output has increased as a result of production intensification, which is directly related to an increase in disease incidence. An important barrier to the sustainability of shrimp production in many nations is the disease outbreaks since the shrimp immune system lacks an adaptive immune system and is entirely dependent on innate immunity to protect it from invasive diseases. One of the most significant aquaculture shrimps in the world is the Pacific white shrimp, *Litopenaeus vannamei*, which has a wide range of salt tolerance, quick growth, and other qualities ideal for intensive farming (Wang et al. 2020).

One of the devastating bacterial illnesses that frequently affects a wide variety of shrimp species is shrimp vibriosis (Valente and Wan 2021). The production and trade of shrimp have become significantly hampered by the bacterial genus *Vibrio*. Vibrio is responsible for a number of illnesses, 100 percent mortality rates, and around 4.5 billion USD losses worldwide (Chellapandian et al. 2021). Even though vibrios are ubiquitous in aquaculture production systems and many are non-pathogenic, which can turn into opportunistic infections when natural defensive systems are disregarded. Shrimp susceptibility to infections is mainly influenced by water quality parameters like pH, salinity, temperature and nutrients in the system. In penaeid shrimp cultured systems, bacterial diseases primarily caused by *Vibrio*, mainly *Vibrio harveyi, V. cholera, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbelli, V. fischeri, V. damsella, V. pelagicus, V. orientalis* etc have been reported (Annam et al. 2015). Severe economic losses because of disease outbreaks due to vibriosis like acute hepatopancreatic necrosis disease (AHPND) was noticed in *Penaeus monodon* and *Penaeus vannamei* cultured farms in Malaysia, India, China, Vietnam, Thailand etc (Schryver et al. 2014).

The application of antibiotics in shrimp industry is very extensive mainly because of three major reasons; susceptibility to pathogens due to their suppressed immune system, least response to vaccination compared to other fishes and their increased demand in global market (Thornber et al. 2020). The increased need for the production of disease-free crustaceans forces the farmers to apply antibiotics intensively in shrimp operation farms. The extensive exposure to antibiotics may definitely lead to the selection pressure and raise antibiotic resistant bacteria in the system (Preena et al. 2020a). Apart from this, plasmid-mediated antimicrobial resistance (AMR) could promote the rapid dissemination of AMR through horizontal gene transfer (HGT) which pose global health threats. Since the shrimps are of significance in food industry, there is increased chance of spread of antimicrobial resistance genes (ARG) from shrimp pathogens to that of humans (Hoelzer et al. 2017). Henceforth, the AMR status in shrimp cultured systems need to be updated through regular detection and monitoring programs for the uniform health of terrestrial and aquatic life beings. Considering the significance of AMR in the shrimp cultured environment, the present study was initiated to reveal the role of shrimp pathogens, especially vibrios in spreading AMR through plasmid curing studies. It has been noticed that cultured shrimps could possibly act as the reservoirs of antibiotic resistant vibrios and found to play a key role in spreading AMR

globally (Costa et al. 2015). Meanwhile, a methodology for determining AMR in vibrios linked to aquatic animals is still in its infancy and needs harmonization, in contrast to the human and terrestrial animal health sectors (Vaiyapuri et al. 2021). The main objective of the current study was to identify and characterize vibrios from infected shrimps (*Litopenaeus vannamei*) of two cultured farms, to compare and ascertain their pattern of antibiotic resistance and to determine whether AMR is of plasmid borne or chromosome mediated.

Materials And Methods

Collection of shrimp samples and processing

The samples for the study were selected from two brackish water shrimp cultured farms (15ppt salinity) located at two different geographical locations of Ernakulam *viz*. Panangad (farm 1) and Paravur (farm 2). Twenty moribund shrimp (*Litopenaeus vannamei*) samples each were collected on ice in sterile containers from two different farms, and were transported aseptically to the lab. The clinical signs noticed in moribund shrimps were recorded.

Isolation Of Vibrios From Infected Shrimps

The hemolymph and hepatopancreas from the infected shrimps of both cultured farms were aseptically homogenized. The processed samples were enriched separately in alkaline peptone water and incubated overnight at 37°C. The serially diluted cultures were inoculated on thiosulphate citrate bile salt sucrose (TCBS) agar medium (Himedia, India) using conventional spread plate technique for the isolation of vibrionaceae groups. The colonies separated were selected on the basis of unique morphology and sub cultured on TCBS agar slants and stored in glycerol at -80°C for further studies. The isolates were then subjected to phenotypic tests such as Gram staining, motility, catalase, oxidase, nitrate reduction, IMViC, urease, gelatin hydrolysis, carbohydrate fermentation tests (glucose, sucrose, lactose, mannose, mannitol and arabinose), TSIA, arginine decarboxylase, ornithine decarboxylase and salt tolerance tests with different salt concentration (0%, 3%,6%,8%,10%,12%,15%,25% and 35%) for the clustering of representative isolates (Cowan and Steel 1974).

Identification Of Clusters Using Ntsys Software

The resulting data based on biochemical characteristics were entered into Excel spreadsheets by designating positive outcomes as “1” and negative results as “0,” and the dendrogram was created using the software NTSYS (Exeter Software, Setauket, NY, USA). Based on similarities, the clusters were categorized and the representative isolates were further studied.

Diversity Determination Using Primer-e Software

Traditional methods for identifying microbial communities involve Shannon- wiener diversity, which directly relates to species richness and evenness. Based on the biochemical characteristics, the diversity index of the bacterial isolates recovered from the shrimps collected from both farms was calculated using the software, Primer E (Clarke and Gorley 2015). Based on the Shannon-weaver diversity index, diversity factors such as species richness, evenness and dominance was also determined.

Molecular Identification And Phylogenetic Analysis

Genomic DNA of the representative isolates generated based on dendrogram has been isolated using salting out method following Miller et al. (2018). Quantity and quality of DNA were confirmed at the absorbance of 260 nm and 280 nm on UV spectrophotometer (Beckman, USA). The 16SrRNA gene PCR analysis was carried out using the primers fD1 (5’CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G3’) and rD1 (5’CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC3’) (Weisburg et al. 1988). The amplified gene products were purified and sequenced using ABI Prism 3700 Big Dye sequencer (SciGenom, Cochin). The 16SrRNA gene sequences were identified by comparing with the sequences in the Genbank database through NCBI BLAST (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov) to assess resemblance to the nearest phylogenetic affiliate in the database. The gene sequences were deposited to Genbank with accession numbers OP019729- OP019739. Phylogenetic analysis was performed using

UPGMA statistical method and Kimura 2-parameter substitution model with Mega X version (Kumar et al. 2018). The tree has been constructed using neighbor-joining method with 1000 bootstrap replications.

Antibiogram Profiling And Determination Of Minimum Inhibitory Concentration Of The Resistant Isolates

The identified isolates had undergone antibiotic susceptibility tests on Mueller–Hinton agar (MHA) (HiMedia, India) using disc diffusion method (CLSI 2020). Thirtyfive antibiotics belonging to 15 classes were selected for ascertaining the antibiotic resistance pattern and is given in Table.1. Lawn culture of isolates were prepared on MHA plates and five antibiotic discs (HiMedia, India) each were placed on the inoculated plates and incubated overnight at 37°C. The size of the inhibition zone (mm) was measured and the results were interpreted as per CLSI guidelines (2020). The multiple antibiotic resistance (MAR) index, ratio of resistant antibiotics to total number of antibiotics exposed, of the isolates was calculated according to the antibiotic susceptibility test results (Krumperman 1983).

The minimum inhibitory concentration (MIC) of antibiotics was also determined for the resistant isolates with MIC strips (Himedia, India) coated with various antibiotic concentration. The tested isolates were maintained at uniform concentration (10^5 CFU/mL) while comparing with the standard, 0.5 McFarland solution. Thereafter, the cultures along with a positive control, CLSI reference strain *E. coli* ATCC 25922, were inoculated on MHA and three MIC strips each were placed on the inoculated plates followed by incubation at 37°C for 24hrs. The results were recorded according to CLSI (2020).

Plasmid Extraction, Curing And Antibiogram Profiling

The plasmids of overnight grown antimicrobial resistant isolates were extracted using plasmid mini preparation kit (Sigma Aldrich, USA). The eluted plasmids were observed on 1% agarose gel electrophoresis and visualized in gel documentation system (Bio-Rad Laboratories, California, USA). The plasmid bearing isolates had undergone plasmid curing using sodium dodecyl sulfate (SDS) (Sigma Aldrich, USA) following Letchumanan et al. (2015). For this, the isolates were grown individually on alkaline peptone broth provided with various concentration (0.1-1mg/ml) of SDS and kept in shaker incubator overnight at 37C. The antibiotic susceptibility tests were performed again to check whether any change in AMR profile after plasmid curing.

Results And Discussion

Isolation of vibrionaceae groups and determination of diversity

As per the farmer's information during sampling, the infected shrimps had shown abnormal swimming, loss of appetite, reddish body discoloration and necrosis of hepatopancreas which lead to the mortality of shrimps from both farms (Supplementary file ESM Fig. 1). More than 100 colonies each were segregated on TCBS agar plates from different parts of shrimp samples of two farms. Majority of the colonies were green colored and others exhibited yellow and black color. Based on the unique features like color, size, shape, margin etc. suspected vibrio colonies were selected from the pooled shrimp samples of both farms. While assessing the salt tolerance of the isolates, all the isolates have grown with NaCl up to 12% and the growth was arrested on further salinity. This indicates that the isolates are halotolerant which might be because they inhabit brackish water environment and however, not halophilic. All the recovered isolates were found as Gram negative and motile. On phenotypic characterization, the isolates have shown typical biochemical features and based on that dendrogram with 5 clusters for farm 1 with a distance coefficient of 0.50 to 1 (Supplementary file ESM Fig. 2) and 6 for farm 2 with 0.48 to 1 (Supplementary file ESM Fig. 3) were generated using NtSys software. Thus the representative 5 isolates from farm 1 and 6 from farm 2 were selected for further characterization.

According to Primer E software, the Shannon wiener diversity index of the recovered isolates was found to be H' (loge) = 1.506 and 1.69 for farm 1 and 2 respectively. This in turn is determined by the factors such as species richness (d = 0.868 for farm 1 and 1.086 for farm 2), evenness (J'= 0.935 for farm 1 and 0.943 for farm 2) and dominance (1-lambda = 0.767 for farm 1 and 0.809 for farm 2). Thus microbial diversity is found to be higher in farm 1 compared to farm 2.

On sequence analysis of the 16SrRNA gene of the representative isolates, *Vibrio alginolyticus*, *V. cholera*, *V. fluvialis*, *V. mimicus* and *Shewanella algae* were identified from the samples of farm 1 and *Vibrio parahaemolyticus*, *V. cholera*, *Aeromonas hydrophila*, *A. salmonicida*, *Citrobacter freundii* and *S. algae* from that of farm 2. Phylogenetic tree (Fig. 1) consisting 5 operational taxonomic units (OTUs) for farm 1 and 6 for farm 2 was constructed on comparison with similar 16rRNA gene sequences from the Genbank databases. The distance coefficient seems to be low for farm 1 and 2 with 0.03 r to 0.0r and 0.04r to 0.0r respectively since the isolates are of related groups. The phylogenetic tree of farm 1 isolates comprised mainly two branches separated at the distance coefficient of 0.03r. All the *Vibrio* sp were clustered together in first branch with *V. cholerae* and *V. mimicus* under one sub-branch separated from *V. fluvialis* and *V. alginolyticus* at 0.02r under another one. While *S. algae* was clustered alone under second branch. While the distance coefficient of phylogenetic tree constructed with farm 2 isolates is 0.04r with main two branches. The aeromonads group and *Shewanella* were grouped under one branch with separation at 0.04r from the second branch consisting *Citrobacter* and vibrios with the similarity coefficient of 0.03r. The overall mean distance, determined using Mega X software, between the 16srRNA gene sequences of farm 1 and farm 2 isolates was found to be 0.04 and 0.07 respectively.

Even though the intention of the present study was to recover vibrios from the infected shrimps, other microbes belonging to aeromonads (*A. hydrophila* and *A. salmonicida*) and enterobacteraceae groups (*Citrobacter freundii*) along with *Shewanella algae* were also resolved on TCBS medium. As noticed in previous studies, although the medium is selective for vibrios, it may promote the growth of other organisms like *Aeromonas, Shewanella, Pseudomonas* etc. and can be easily misinterpreted as *Vibrio* by showing similar morphology and biochemical test results (Canellas et al. 2021). *Shewanella* was also reported to grow on TCBS medium as black colored colonies (Wang et al. 2009) while on sub culturing it was difficult to get differentiated from vibrios in our study. Hence molecular identification became essential for the genus and species level confirmation.

The most predominant organism isolated from all the infected parts of shrimps belonging to both farms were *Vibrio cholera*. It was followed by *V. alginolyticus* and *V. fluvialis* in the case of farm 1 and *V. parahaemolyticus* from farm 2. While the number of colonies of *Aeromonas* and *S. algae* recovered from the infected samples was less. It is reported that non-vibrio species like *Aeromonas* and *Shewanella* could grow on standard TCBS medium but at lower growth rate compared to vibrios (Valente and Wan 2021). The diversity of *Vibrio* sp. recovered from farm 1 was high compared to farm 2 while the latter showed increased overall diversity. The vibrios segregated from both farms such as *V. cholerae, V. alginolyticus, V. parahaemolyticus, V. fluvialis* and *V. mimicus* were noticed to cause significant diseases in decapod crustaceans (Valente and Wan 2021). Most of the vibriosis causing bacteria are found to be opportunistic in shrimps and prawns and could induce infections when environmental stresses enhanced. Even though *V. cholera* is ubiquitous in aquatic environments, it was observed to cause severe infections like yellowing of legs and white feces syndrome in fresh water cultured white leg shrimps (*Penaeus vannamei*) (Cao et al. 2015). Hepatopancreatic necrosis disease induced by *V. alginolyticus* and *V. parahaemolyticus* is severe and prevalent in all kinds of cultured shrimps (Morales-Covarrubias et al. 2018). In juveniles and adults of crustaceans, shell diseases caused by *V. fluvialis*, *V. mimicus*, *V. alginolyticus* and *V. parahaemolyticus* are also relevant in severe economic losses (Valente and Wan 2021). In addition to vibrios, *A. hydrophila* was also recently reported as an emerging pathogen in causing mass mortalities of *Litopenaeus vannamei* (Zhou et al. 2019). Hence the occurrence of aeromonads within the infected shrimps might also have contributed pathogenicity to the shrimps. The coinfection of pathogenic *Aeromonas*, *Vibrio* and *Citrobacter* in crustaceans were already reported in earlier studies (Thancharoen et al. 2019) and which is in close agreement with the present study. Another isolate which was resolved from both farms was *S. algae* and was reported for the first time by Cao et al. (2018) as emerging pathogen in causing black spot disease in white leg shrimps. Despite the ubiquitous occurrence of *S. algae* in high salinity aquatic environments, the reports of shrimps with this species infection is less; while a novel species, *S. khirikhana* was recently isolated from shrimps in causing early mortality syndrome by Prachumwat et al. (2020). Hence more studies are essential to recover novel species from crustaceans utilizing appropriate selective media for the pathogens. Since diverse species of vibrios and other related pathogens were recovered from the infected shrimps, their response towards different classes of antimicrobials need to be monitored for the effective treatment and management measures.

Antibiogram Profiling

The antimicrobial resistance pattern of the isolates from farm 1 revealed that they were resistant towards a maximum of 21 and a minimum of 9 antibiotics and those from farm 2 showed a maximum of 15 and minimum of 9. The resistance and sensitivity pattern of all the isolates resolved from both shrimp farms towards 35 tested antibiotics are represented as heat map in Fig. 2. Out

of the 35 antibiotics tested, all the isolates were found to be resistant to tetracycline and ampicillin and all were sensitive to erythromycin, ciprofloxacin and cefazolin. Besides, amikacin, meropenem and tobramycin were found to ineffective against all the isolates from farm 1 and vancomycin and all the quinolones group were ineffective for any of the isolates from farm 2. The details showing the percentage of isolates from both farms exhibiting resistance towards 15 different antibiotic classes are given in Table.1 and figures are depicted in Fig. 3. In the case of isolates from farm 1, hundred percent of the isolates have shown AMR towards betalactams, first and third generation cephalosporins and tetracyclines and more than fifty percent towards carbapenems, aminoglycosides, quinolones and phenicols; while least percent to macrolides, glycopeptides, nitrofurans, macrolides and sulphonamides. While, those from farm 2 exhibited hundred percent resistance to beta lactams and tetracyclines and greater than fifty percent towards all the other classes except glycopeptides and first and third generation cephalosporins.

Amidst the 15 classes tested, the isolates from farm 1 possessed AMR towards at least one antibiotic of minimum 8 classes and a maximum of 12, while those from farm 2 have shown minimum of 7 and maximum of 12 classes. The graph showing the percentage of AMR shown by each isolate from the samples of both farms towards different classes of antibiotics is given in Fig. 3. The most resistant isolate recovered from the shrimp samples was *V. alginolyticus*, recovered from farm 1 sample, by showing resistance towards 21 antibiotics belonging to 11 different classes with the MAR index of 0.6. It is to be pointed out that they were found to be insusceptible towards all the new generation cephalosporins and is challenging. The second most resistant isolate, *V. mimicus* was also recovered from the farm 1 shrimps with the MAR index of 0.54 by exhibiting AMR towards 19 antibiotics of 12 different classes. This is followed by *V. fluvialis* from farm 1 and *V. parahaemolyticus* from farm 2 with the MAR index of 0.43 by expressing resistance towards 15 antibiotics of 8 and 9 various classes respectively. The least resistant isolates *viz*. *Shewanella algae* and *Vibrio cholera* segregated from farm 1 were found to have the MAR index of 0.34 and 0.25 respectively. Meanwhile, *V. cholerae* isolated from farm 2 displayed MAR index of 0.34 followed by 0.31 for *A. hydrophila* and 0.28 for *A. salmonicida*. However, *C. freundii* from farm 2 showed resistance towards 13 antibiotics of 9 different classes with the MAR index of 0.37. The most sensitive isolate compared to other tested ones was *S. algae* from farm 2 with the MAR index of 0.35. Thus all the isolates recovered from both farms exhibited MAR index of > 0.2, which highlighted the increased contamination of antibiotic residues in both systems (Preena et al. 2020b). The minimum inhibitory concentration of most of the resistant antibiotics was found to be higher than 256mcg/ml and the details are given in supplementary file ESM.Table.1 and Table. 2.

Since *V. alginolyticus* is one of the most significant pathogens in shrimp cultured systems, their resistance towards multiple drugs with higher MAR index raise major concerns. Multiple antibiotic resistant *V. alginolyticus* is already reported as a worldwide threatening species to human and animal health and food safety (Yin et al. 2022). Yasir et al. (2020) reported the draft genome sequence of *V. alginolyticus* from marine system, which was found to comprise multiple ARG linked with multidrug resistant efflux pumps and highlighted their major role in AMR dissemination. In addition to *V. alginolyticus*, *V.parahaemolyticus* is also a significant zoonotic pathogen found among the shrimps and hence their multidrug resistance also presents potential risks to human health (Ina-Salwany et al. 2019). The existence of AMR among *V. parahaemolyticus* isolates is evidenced in several studies and pointed out their role as reservoirs of ARG in aquaculture industry (Reyhanath et al. 2014; Letchumanan et al. 2015). Similar to the present study, Banerjee et al. (2012) demonstrated the coexistence of multidrug resistant *V.alginolyticus, V.cholerae, V.parahaemolyticus* and *V.mimicus* along with other pathogens in diseased cultured *Litopenaeus vannamei*. Most of the vibrios isolated in the study, especially *V.cholerae, V.parahaemolyticus* and *V.fluvialis* are usually considered as opportunistic pathogens and reported to cause disease outbreaks when shrimps are stressed and also found to be responsible for major food borne and zoonotic diseases (Kitiyodom et al. 2010). Hence the presence of enhanced antimicrobial resistance in such zoonotic pathogens pose threats even to human health.

Prevalence of antibiotic resistance in virulent aeromonads, especially *A. hydrophila* among the cultured fishes, ornamental fishes and crustaceans were noticed in several studies earlier and is in accordance with this study (Vivekanandhan et al. 2002; Preena et al. 2019a, b; Preena et al. 2020c; Preena et al. 2021). However, although *A. salmonicida* is found to cause profound infections, like furunculosis, in salmonid fish, it is least reported in fresh water or brackish water fish shrimps. Likewise, infection of shrimps with *Citrobacter* and *Shewanella* is also least reported. Hence it is surprising to notice the recovery of those multi drug resistant strains from shrimps under study and need further characterization. Nevertheless, their antimicrobial resistance pattern indicated that the prevalence of such least reported bacteria also play a key role in spreading AMR among the shrimp pathogens. Higher MAR index of putative pathogen, *S. algae* was recently reported from marine environments also corroborated with the present study (Ibrahim et al. 2021). Even though diverse bacteria were recovered and an overall increased AMR and uniform resistance pattern were observed

among the samples from farm 2 compared to farm 1, the MAR index was found to be higher in the case of farm 1 with *V. alginoyliticus* and *V. mimicus* as the most resistant ones.

Plasmid Curing And Antimicrobial Susceptibility Studies

On analyzing the plasmids for determining the occurrence of ARG, all the isolates were found to have plasmids (> 10kb). Hence all the isolates were treated with various concentration of SDS and 0.2mg/ml was found to be optimum for plasmid curing for every isolate. The high frequency of plasmid elimination efficiency of SDS was reported in the earlier period onwards (Tomoeda et al. 1974) and because of the safe handling and easy disposal compared to ethidium bromide and acridine orange, SDS was selected for the present study (Letchumanan et al. 2015). After curing, plasmid elimination was confirmed by re-extraction followed by visualization on 1% agarose gel and assured that cells were devoid of plasmids. Altered resistance phenotype was observed following repeated antimicrobial sensitivity tests and a drastic change in MAR index was noticed for all organisms. *V. cholerae* and *V. mimicus* from farm 1 have lost antimicrobial resistance completely after plasmid elimination and sensitive towards all antibiotics. *V. alginolyticus, V. fluvialis* and *S. algae* were also found to be susceptible to all antibiotics except one or two antibiotics and exhibited a marked changed in MAR index. The case is not different with farm 2 also, where all aeromonads and *Shewanella* possessed the MAR index of 0 after plasmid curing. *V. parahaemolyticus*, *V. cholerae* and *C. freundii* also showed a great alteration in resistance phenotype. The antimicrobial resistance phenotype along with MAR index before and after plasmid curing is given in Table.2. The information generated denotes that most of the antimicrobial resistance genes in majority of the isolates from both farms are of plasmid borne. Even the samples were collected from two different geographic locations, an increased antimicrobial resistance pattern was observed ubiquitously. This might be due to the global indiscriminate usage of prophylactic drugs and possible rapid AMR spread through horizontal gene transfer.

It is well known reported that most of the vibrios harbor mobile genetic elements like plasmids, integrons, integrating conjugative elements, transposons etc which could acquire genetic determinants of AMR (Dutta et al. 2021). *Aeromonas* and *Citrobacter* were noticed to possess plasmids of various sizes and are well known for their capability to acquire multiple drug resistance genes within their mobile genetic elements (Fang et al. 2021).The horizontal movement of antibiotic resistance gene harboring plasmids between *A. hydrophila* and other strains like *E.coli* was demonstrated in previous studies and bring out their significance in rapid dissemination of AMR from aquatic to terrestrial environment through fish food (Stratev and Odeyemi 2016). The mobilizing capacity of multidrug resistance genes in *S. algae* and various vibrio strains and their active role as the reservoir of AMR in aquaculture farms and further spread to clinical pathogens was clearly determined through resistome analysis by Zago et al. (2020). It is also stated that antimicrobial resistant pathogens, especially *Vibrio* and *Aeromonas* released in to the shrimp cultured ponds have the capability to become predominant strains in the system, which raise the sudden chance of AMR circulation (Thornber et al. 2020). In addition, virulence of pathogens can be further enhanced by the integration of antimicrobial resistance gene harboring plasmids, which highlights the contribution of ARGs in causing diseases (Yu et al. 2012). Thus our study is in line with the previous studies proving the higher antimicrobial resistance index of the isolated vibrios and other bacteria and their potential in spreading diseases and AMR to the whole ecosystem.

As discussed earlier, due to the excess of use of antibiotics, shrimp aquaculture system could act as one of the major natural hub of multi drug resistance genes. It is evident in a recent study through advanced high-throughput sequencing techniques that there exists a strong correlation between bacterial diversity, antimicrobial resistance genes and mobile genetic elements in shrimp aquaculture environments (Fang et al. 2021). The prevalence of AMR among the shrimp pathogens raise major concerns to the human health also through the consumption of such under cooked shrimps. There is also a chance of exchange of ARG from aquatic bacteria to human gut microbiome and further to clinical pathogens through HGT thereby establishing AMR to a great extent in humans and make the treatment difficult (Thornber et al. 2020). This is exemplified by the laboratory conjugation experiments in which high transfer frequency of ARG from marine strains to human gut flora like *E. coli* has been demonstrated (Pepi and Focardi 2021). It can be concluded that the shrimp cultured system could serve as active “hotspots” of ARGs and shrimps as delivery vehicle in contributing AMR from aquatic pathogens to human. In this scenario, the attempt of this present study is of utmost importance to unravel the mode of transfer of AMR among the pathogens in shrimp cultured systems. Hence appropriate investigation systems and continuous surveillance programs need to be implemented for the timely detection of antibiotic resistant pathogens and adoption of preventive measures. Thus the fight against global AMR menace can be successfully overcome by considering the uniform fitness of all living beings under “One-Health” umbrella.

Conclusion

Multi-drug resistant vibrios and other pathogens were isolated from infected shrimps of different farms. All the isolates possessed MAR index > 0.2 and majority exhibited MIC > 256mcg/ml thereby indicating the excess exposure of antibiotics in the systems. Every recovered isolate was resistant towards tetracycline and ampicillin and sensitive towards erythromycin, ciprofloxacin and cefazolin. Antimicrobial resistance shown by the studied isolates towards new generation cephaloporins is challenging. Plasmid curing studies revealed that AMR possessed by most of the isolates is of plasmid mediated. This plasmid-borne AMR genes can be easily transferred to other aquatic and non-aquatic pathogens through lateral gene transfer, raising global AMR spread.

Declarations

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Conflict of Interest

The authors have no conflict of interest to declare.

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Author's contributions statement

Following contributions had been done by the authors

Conceived of or designed study: P.G Preena

Performed research: Prabina Das, Sowmya. P. Mohandas, J.C Anjana and P.G Preena

Data analysis and interpretation: P.G Preena, Prabina Das, Sowmya. P. Mohandas and J.C Anjana

Manuscript draft: Prabina Das, P.G Preena, T. Raja Swaminathan and K Manjusha.

All authors read and approved the final manuscript.

Data availability

The datasets generated during the current study are available from the corresponding author on reasonable request.

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Tables

Table.1 Selected antibiotic and the percentage of isolates from farm1 and farm2 shrimp samples showing AMR towards different antibiotics

Antimicrobials	Disc content (µg)	% of resistant isolates from infected shrimps	
		Farm 1	Farm 2
β-Lactams			
Amoxicillin (AMX)	25	20	33.3
Amoxicillin/clavulanic acid (AUG)	25	20	0
Ampicillin (AMP)	25	100	100
Aztreonam (AT)	30	40	50
Piperacillin (PI)	100	80	
First generation cephalosporins			
Cefazolin (CZ)	30	0	0
Cefuroxime (CXM)	30	80	66.6
Cephalothin (CEP)	30	60	33.3
Second generation cephalosporins			
Cefoxitin (CX)	30	20	33.3
Third generation cephalosporins			
Cefixime/clavulanic acid (CMC)	5	80	50
Cefoperazone (CPZ)	75	60	33.3
Cefotaxime (CTX)	30	60	16.66
Ceftazidime (CAZ)	30	80	50
Ceftriaxone (CTR)	30	80	33.3
Fourth generation cephalosporins			
Cefepime (CPM)	30	40	33.3
Carbapenem			
Doripenem (DOR)	10	40	50
Imipenem (IPM)	10	20	16.66
Meropenem (MRP)	10	0	16.66
Aminoglycosides			
Amikacin (AK)	30	0	16.66
Gentamycin (HLG)	30	40	33.3
Streptomycin (S)	10	40	66.6
Tobramycin (TOB)	10	0	33.3
Macrolides			
Azithromycin (AZM)	30	20	33.3
Erythromycin (E)	15	0	0
Quinolones and Fluoroquinolones			

Ciprofloxacin (CIP)	30	0	0
Enrofloxacin (EX)	10	60	0
Levofloxacin(LE)	5	40	0
Norfloxacin (NX)	10	40	0
Tetracyclines			
Oxytetracycline (O)	30	100	100
Sulphonamides			
Co-trimoxazole (COT)	25	40	50
Phenicols			
Chloramphenicol (C)	30	80	66.6
Nitrofurans			
Nitrofurantoin (NIT)	100	40	33.3
Glycopeptides			
Vancomycin (VA)	30	20	0
Other antibiotics			
Polymyxin-B (PB)	30	20	33.3
Rifampicin (RIF)	5	40	50

Table.2 Antibiogram profile and MAR index of the isolates from the shrimp samples before and after plasmid curing

Species	Genbank accession no.	Antibiogram phenotype profile	MAR index	Antibiogram phenotype profile	MAR index
		Before plasmid curing		After plasmid curing	
Farm 1					
Vibrio alginolyticus	OP019731	Amoxicillin, ampicillin, aztreonam, azithromycin, cefoperazone, cefotaxime, co-trimoxazole, polymyxin, cefixime, ceftriaxone nitrofurantoin, piperacillin, tetracycline, cefuroxime, cefixime, levofloxacin, norfloxacin, enrofloxacin, cefoxitin, ceftazidime, cephalothin	0.60	Cefixime, aztreonam	0.05
Vibrio cholerae	OP019729	Ampicillin, tetracycline, enrofloxacin, cefoperazone, gentamycin, chloramphenicol, doripenem, ceftazidime, cefuroxime	0.25	Nil	0
Vibrio fluvialis	OP019730	Ampicillin/ tetracycline/ cefotaxime/ ceftriaxone/ piperacillin/ceftazidime/amoxicillin-clavulanic acid/cefixime/rifampicin/cefoperazone/doripenem/ levofloxacin/aztreonam/chloramphenicol/cephalothin	0.24	chloramphenicol	0.02
Vibrio mimicus	OP019732	Cefepime, cefuroxime, cefotaxime, ceftriaxone, piperacillin, ceftazidime, rifampicin, cefoperazone, vancomycin, polymyxin-B, ampicillin, aztreonam, cefixime, tetracycline, chloramphenicol, gentamycin, streptomycin, imipenem, nitofurantoin	0.54	Nil	0
Shewanella algae	OP019733	Ampicillin, tetracycline, cefuroxime, ceftriaxone, streptomycin, enrofloxacin, cefexime, piperacillin, chloramphenicol, cephalothin, norfloxacin, co-trimoxazole	0.34	Enrofloxacin	0.02
Farm 2					
Vibrio cholerae	OP019734	Ampicillin, tobramycin, amikacin, amoxicillin, cefexime, streptomycin, gentamycin, tetracycline, chloramphenicol, imipenem, doripenem, cephalothin	0.34	Amoxicillin, chloramphenicol	0.05
Vibrio parahaemolyticus	OP019735	Ampicillin, co-trimoxazole, cefexime, ceftriaxone, tetracycline, nitrofurantoin, polymyxin-B, ceftazidime, aztreonam, azithromycin, rifampicin, cefotaxime, cefoperazone, tobramycin, cefepime	0.42	Streptomycin	0.02
Citrobacter freundii	OP019736	Aztreonam, streptomycin, ceftriaxone, nitrofurantoin, ampicillin, tetracycline, polymyxin-B, cefepime, ceftazidime, cefexime, cefuroxime, cefoperazone, cefoxitin	0.37	Aztreonam, polymyxin-B	0.05
Aeromonas hydrophila	OP019737	Ampicillin, tetracycline, rifampicin, piperacillin, streptomycin, cephalothin, cefuroxime, doripenem, chloramphenicol, cefoxitin, ceftazidime	0.31	Nil	0
Aeromonas salmonicida	OP019738	Amoxicillin, ampicillin, tetracycline, meropenem, co-trimoxazole, cefuroxime, amikacin, azithromycin, doripenem, chloramphenicol	0.28	Nil	0
Shewanella algae	OP019739	Streptomycin, rifampicin, tetracycline, co-trimoxazole, ampicillin, aztreonam, cefuroxime, gentamycin, chloramphenicol	0.25	Nil	0

Figures

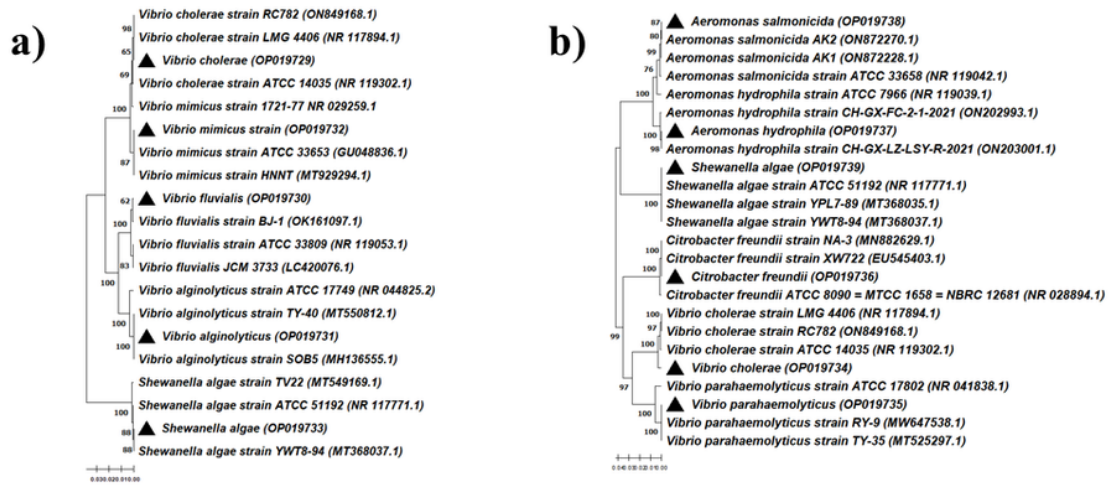

Figure 1

Phylogenetic tree constructed using Mega X with 16SrRNA sequences of isolates recovered from shrimp samples of a) farm 1 and b) farm 2.

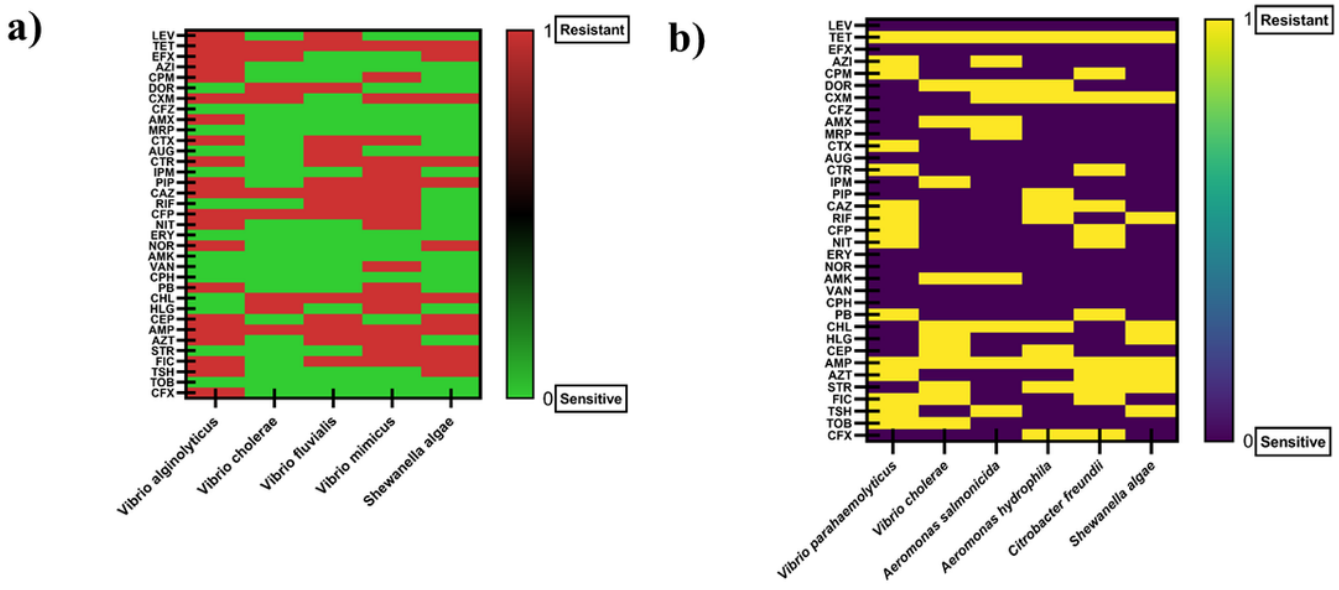

Figure 2

Heat map showing antibiogram profiles of shrimp isolates from a) farm 1 and b) farm 2 towards 35 antibiotics.

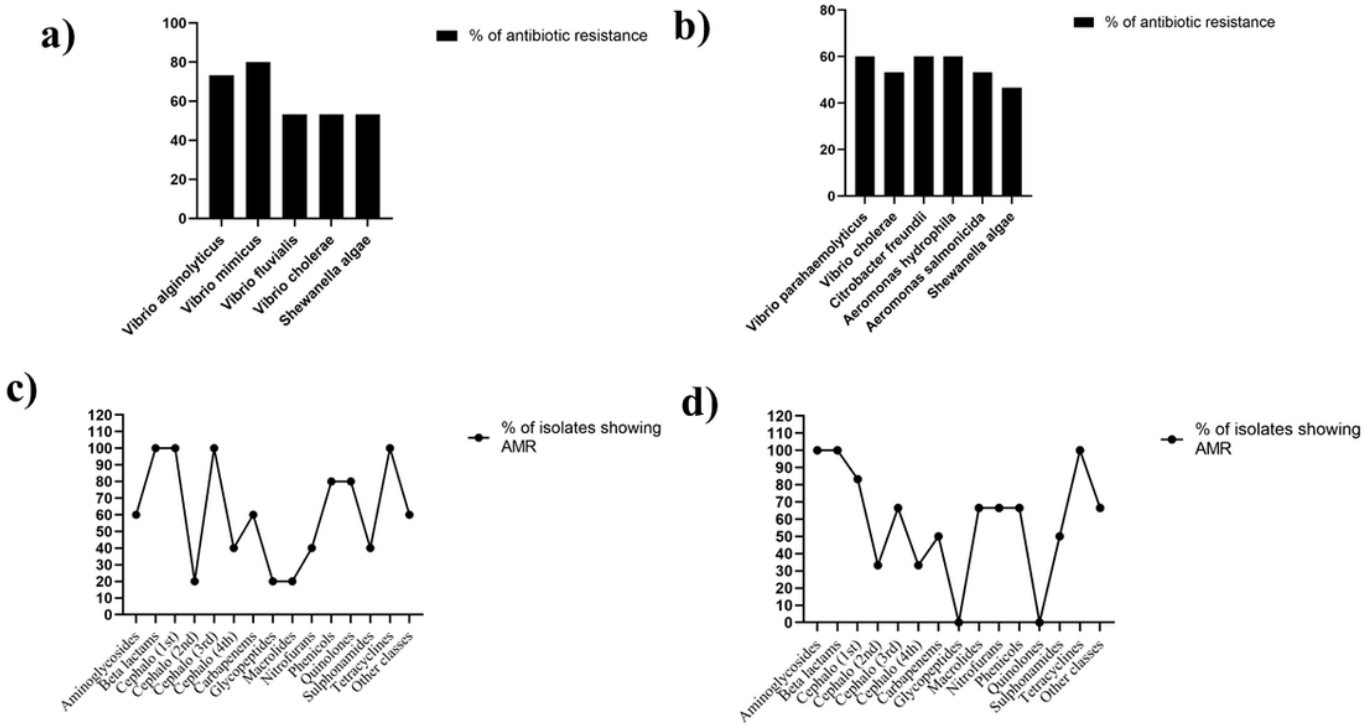

Figure 3

The graph showing the percent of AMR possessed by shrimp isolates from a) farm 1 and b) farm 2 and the percent of shrimp isolates from c) farm 1 and d) farm 2, showing AMR towards 15 antimicrobial classes.

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